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Isolation and Identification of *Aspergillus* Section *Fumigati* Strains from Arable Soil in Korea

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63 strains of *Aspergillus* section *Fumigati* were isolated from 17 samples of arable soil in a central province of Korea. Based on the results of genotypic and phenotypic analyses, they were identified as *Aspergillus fumigatus*, *A. lentulus*, *Neosartorya coreana*, *N. fennelliae*, *N. fischeri*, *N. glabra*, *N. hiratsukae*, *N. laciniosa*, *N. pseudofischeri*, *N. quadricincta*, *N. spinosa* and *N. udagawae*. Among these, *N. fennelliae*, *N. hiratsukae*, *N. quadricincta*, and *N. udagawae* had not been previously recorded in Korea. The diversity of *Aspergillus* section *Fumigati* species from arable soil in Korea is also addressed.

KEYWORDS : *Aspergillus* section *Fumigati*, Korea, *Neosartorya*, Soil

Aspergillus section *Fumigati* (AsF) is an economically important fungus and teleomorphic species of this section belong to the genus *Neosartorya*. Eight strictly mitotic species and 17 *Neosartorya* species in AsF are generally accepted [1].

The diversity of AsF species in Korean soil remains poorly characterized. *Aspergillus fumigatus* has generally been reported in paddy fields [2-6] and forest soils [7-9] and *Neosartorya fischeri* has been detected primarily in paddy fields [2, 6]. On the contrary, *A. fumigatus* has been frequently studied in clinical environments in Korea [10-12]. Recently, Hong *et al.* [13] reported two new species--*Neosartorya coreana* and *N. laciniosa*--in Korean soil, with Korean strains of *N. fischeri*, *N. glabra*, *N. pseudofischeri*, and *N. spinosa*. Hong *et al.* [14] also isolated *Aspergillus lentulus* from Korean soil.

In this study, we isolated AsF strains from arable soil in a central province of Korea, and identified their species by genotypic and phenotypic characteristics in order to evaluate the diversity of AsF in Korean soils.

Materials and Methods

Soil samples. Soil samples were collected from 17 arable soil sites. Their geographical origins and crops are listed in Table 1. The soil samples were preserved at 4°C

Table 1. List of arable soil samples in Korea used in this study

Sample no.	Geographical Origin	Crop	Remark
14	Daejeon	*Glycine max*	
15	Daejeon	*Allium fistulosum*	
16	Daejeon	*Perilla frutescens*	
17	Daejeon	*Lycopersicon esculentum*	
18	Daejeon	*Helianthus annuus*	
19	Daejeon	*Capsicum annuum*	
20	Yeongi, Chungnam	*Sesamum indicum*	
21	Yeongi, Chungnam	*Capsicum annuum*	
22	Yeongi, Chungnam	*Zea myas*	
23	Buyeo, Chungnam	*Lycopersicon esculentum*	
24	Buyeo, Chungnam	*Lycopersicon esculentum*	Infected with *Fusarium* sp.
25	Buyeo, Chungnam	*Lycopersicon esculentum*	
26	Buyeo, Chungnam	*Lycopersicon esculentum*	Infected with *Fusarium* sp.
27	Buyeo, Chungnam	*Lycopersicon esculentum*	Healthy but surrounded by *Phytophthora* diseased tomato
28	Buyeo, Chungnam	*Lycopersicon esculentum*	Infected with *Phytophthora* sp.
29	Chungju, Chungbuk	*Capsicum annuum*	Infected with *Phytophthora* sp.
30	Chungju, Chungbuk	*Capsicum annuum*	

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until analysis.

Isolation of *Aspergillus* strains. From each soil sample, 10 g of soil was suspended in 90 mL of sterilized water in a 250 mL flask and was shaken for 30 min at 200 rpm. Two isolation methods were utilized for each soil sample. To screen the xerophilic strains, the suspensions were diluted further by factors of 10. One mL aliquots of the suspensions (10^{-1} to 10^{-3}) were pipetted into 9 cm Petri dishes and mixed with 20 mL of undercooled (ca. 45°C) Dichloran 18% Glycerol Agar (31.5 g Dichloran-Glycerol Agar Base [Oxoid CM0729], 220 g glycerol, 0.1 g chloramphenicol, 1 L distilled water). To screen thermo-tolerant strains (for ascospores of *Neosartorya*), the suspensions were incubated in water baths at 75°C for 30 min. 25 mL heat-treated suspensions were pipetted into 14.5 mm Petri dishes and mixed with equal volumes of undercooled (ca. 45°C) MEA ds-chloramphenicol (50 mg/L). The plates were incubated for 2~4 days for the screening of xerophilic strains, and for 7~21 days for thermotolerant strains. Fungal colonies which showed typical *Aspergillus*, *Penicillium* and their teleomorphic morphology under a stereo-microscope were transferred into malt extract agar (MEA), and incubated for 7~21 days, after which their genera were

Table 2. *Aspergillus* section *Fumigati* strains used in this study

Species	KACC no.	Source[a]	RAPD[b]	β-tubulin[c]	Species	KACC no.	Source[a]	RAPD[b]	β-tubulin[c]
A. fumigatus	41388	14-10		AY685147	*N. glabra*	41656	26-24	41654	
A. fumigatus	41389	30-19	41388		*N. glabra*	41617	CBS 111.55^T		AY870734
A. fumigatus	41390	28-11	41388		*N. hiratsukae*	41688	24-11		DQ534095
A. fumigatus	41143	CBS 133.61		AY685150	*N. hiratsukae*	F3774	26-44	41688	
A. lentulus	41391	22-1		AY685170	*N. hiratsukae*	41689	26-36		DQ534096
A. lentulus	41392	15-6		AY685171	*N. hiratsukae*	41692	16-7		DQ534097
A. lentulus	41393	19-26		AY685172	*N. hiratsukae*	41693	26-1	41692	
A. lentulus	41681	29-33	41393		*N. hiratsukae*	41127	CBS 294.93^T		AF057324
A. lentulus	41682	30-16	41393		*N. laciniosa*	41652	16-1		AY870747
A. lentulus	41642	22-7		AY818358	*N. laciniosa*	41658	27-6	41657^T	
A. lentulus	41394	25-19		AY685173	*N. laciniosa*	41660	19-10	41657^T	
A. lentulus	41395	30-8		AY685174	*N. laciniosa*	41657^T	28-13		AY870756
A. lentulus	41940^T	CBS 117887^T		AY738513	*N. pseudofischeri*	41653	26-7		AY870740
N. coreana	41659^T	23-5		AY870758	*N. pseudofischeri*	41661	16-12		AY870741
N. fennelliae a	41675	29-17		DQ534082	*N. pseudofischeri*	41690	27-4	41661	
N. fennelliae A	41676	27-9		DQ534083	*N. pseudofischeri*	F3800	28-9	41661	
N. fennelliae A	41677	16-2	NP	DQ534084	*N. pseudofischeri*	41128	CBS 208.92^T		AY870742
N. fennelliae a	41678	28-16	41675		*N. quadricincta*	F3749	26-14		DQ534098
N. fennelliae a	41679	26-42	41675		*N. quadricincta*	F3768	26-13	F3749	DQ534099
N. fennelliae a	F3743	29-53		DQ534085	*N. quadricincta*	F3786	24-13		DQ534100
N. fennelliae A	41680	21-14	NP	DQ534086	*N. quadricincta*	F3809	26-2	F3810	
N. fennelliae A	F3746	30-11		DQ534087	*N. quadricincta*	F3810	26-29		DQ534101
N. fennelliae A	F3747	22-9		DQ534088	*N. quadricincta*	41694	26-34	F3810	
N. fennelliae A	F3748	16-21		DQ534089	*N. quadricincta*	F3812	26-35	F3810	
N. fennelliae A	F3760	23-9		DQ534090	*N. quadricincta*	41695	25-1	F3810	
N. fennelliae a	F3762	29-15		DQ534091	*N. quadricincta*	41696	18-9	F3810	
N. fennelliae a	F3763	29-27		DQ534092	*N. quadricincta*	41173	CBS 135.52^T		AF057326
N. fennelliae a	F3764	29-35	F3763		*N. spinosa*	41662	25-3		AY870725
N. fennelliae a	F3765	16-3	NP	DQ534093	*N. spinosa*	41663	19-5	41662	
N. fennelliae a	41687	17-3	NP	DQ534094	*N. spinosa*	41162	CBS 483.65^T		AF057329
N. fennelliae a	41150	CBS 599.74^T		DQ114128	*N. udagawae*	41683	22-16		DQ534102
N. fennelliae A	41125	CBS 598.74^T		DQ114127	*N. udagawae*	41684	19-33	41683	
N. fischeri	41664	20-11		AY870733	*N. udagawae*	F3756	19-32	41683	
N. fischeri	41665	19-14	41664		*N. udagawae*	F3759	22-12	41683	DQ534103
N. fischeri	41182	CBS 544.65^T		AF057322	*N. udagawae*	41155	CBS 114217^T		AF132226
N. glabra	41654	23-6		AY870737	*N. udagawae*	41156	CBS 114218^T		AF132230
N. glabra	41655	28-6	41654		*Neosartorya* sp.	41691	28-7		DQ114123

[a]Front two digits in the numbers correspond to the sample number in Table 1.
[b]In random amplified polymorphic DNA (RAPD)-PCRs, the strain showed the same band patterns with the strain written in RAPD column.
[c]GenBank accession number of β-tubulin gene.
CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; KACC, Korean Agricultural Culture Collection, Suwon, Korea; ^{T}, type strain; NP, not performed.

determined on the basis of macromorphology. Among them, strains of AsF and its teleomorph *Neosartorya* were inoculated at three points on MEA, Czapeks agar (CZA), and oatmeal agar (OA), then incubated for 7~21 days. Their morphological features were evaluated in detail by stereo- and compound microscopy, and strains that evidenced characteristics identical to the other strains were removed. Finally, 63 strains were selected, and are listed in Table 2. They were transferred to MEA slants, incubated, and stored at 15°C until use. The strains were then lyophilized and preserved in the Korean Agricultural Culture Collection (KACC in National Agrobiodiversity Center, NAAS, RDA, Korea), and are publicly available for research.

Species identification. For morphological identification, micro- and macro-morphology analyses were conducted and SEM was also carried out to identify teleomorphic species, according to the method of Hong *et al.* [14]. To evaluate heterothallism, conidial strains were crossed with one another on OA and MEA media and in all combinations with heterothallic strains of *Neosartorya fennelliae*, CBS 598.74^{T} (MT A) and CBS 599.74^{T} (MT a), *N. udagawae*, CBS 114217^{T} and CBS 114218^{T}, *N. spathulata*, CBS 408.89^{T} (MT A) and CBS 406.89^{T} (MT a), with 28 days of incubation at 25°C.

For molecular identification, modified versions of the methods of Hong *et al.* [14] were utilized. Random amplified polymorphic DNA (RAPD)-PCRs with the primers PELF and URP1F were conducted for 63 strains, and 38 genetically different strains were selected. Their β-tubulin genes were sequenced and their sequences were analyzed with the type strains of related species to determine the taxonomic positions of the Korean strains.

Table 3. Composition of fungal strains isolated from 17 samples of arable soil in Korea

Fungi	No. of isolates
Teleomorphic genera	199
Aspergillus	111
Penicillium	79
Paecilomyces	14
Unidentified	37
Total	440

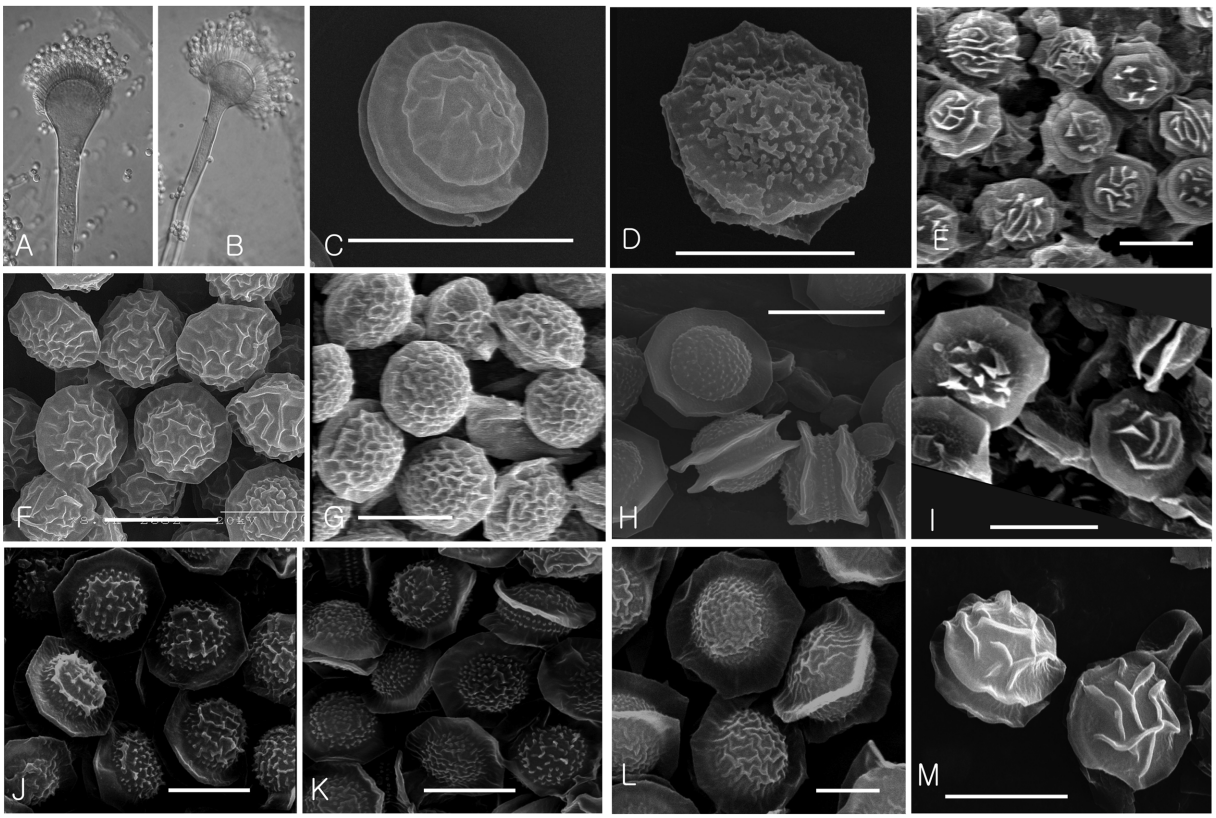

Fig. 1. Aspergillum (A, B) of strict anamorphic species and ascospores (C~M) of *Neosartorya* spp. in *Aspergillus* section *Fumigati* from arable soil in Korea (scale bar = 5 μm). A, *Aspergillus fumigatus* (Korean Agricultural Culture Collection [KACC]) 41388); B, *A. lentulus* (KACC 41642); C, *Neosartorya fennelliae* (KACC 41675 X Centraalbureau voor Schimmelcultures [CBS] 598.74^{T}); D, *N. udagawae* (KACC 41683 X CBS 114218^{T}); E, *N. quadricincta* (KACC F3810); F, *N. fischeri* (KACC 41664); G, *N. hiratsukae* (KACC 41688); H, *N. glabra* (KACC 41654); I, *N. pseudofischeri* (KACC 41661); J, *N. spinosa* (KACC 41663); K, *N. laciniosa* (KACC 41658); L, *N. coreana* (KACC 41659); M, *Neosartorya* sp. (KACC 41691).

Results and Discussion

Mycoflora of arable soil in Korea. Mycoflora in arable soil were analyzed via the dilute plate technique with DG-18 and heat treatments. 440 strains were isolated from 17 arable soil samples. The composition of the strains is presented in Table 3. Strains of AsF and its teleomorph, *Neosartorya* were screened via macromorphology on MEA, CZA, and OA and micromorphology, and 63 strains were ultimately selected (Table 2). The phenotypic (Figs 1 and 2) and genotypic (Fig. 3) analyses allowed the 63 strains to be identified as *Aspergillus fumigatus*, *A. lentulus*, *Neosartorya coreana*, *N. fennelliae*, *N. fischeri*, *N. glabra*, *N. hiratsukae*, *N. laciniosa*, *N. pseudofischeri*, *N. quadricincta*, *N. spinosa* and *N. udagawae*.

Strict anamorph species in AsF. *Aspergillus fumigatus* and *A. lentulus* were isolated in this study. *A. fumigatus* was readily identified on the basis of its dark green to turquoise color and velutinous colony, and fast and abundant conidiation on MEA and Czapek yeast extract agar (CYA). Vesicles (15~23[25] μm) and stipe (6~8[10] μm) widths of species from Korean soils were generally larger than those of the *A. lentulus* strains. The species were isolated from almost all of the examined soil samples.

A. lentulus strains from Korea evidenced slower growth, slower conidiation, thinner stipes (4~6 μm), and more globose vesicles than were observed in *A. fumigatus* (Fig. 1). Seven strains of *A. lentulus* isolated from Korean soil grew at 10°C, but did not grow at 50°C. In our analysis of β-tubulin phylogeny, the strains were found to be clustered with the type strain of *A. lentulus*, CBS 11787 (Fig. 3). The species has been reported only from clinical environments [14]. Interestingly, it was isolated from 7 of 17 soil samples, with a very high frequency (Table 2). This result indicates that *A. lentulus* can frequently be isolated from soil as well as the clinical environment, and its ecological niche could be in soil. Differentiation between *A. fumigatus* and *A. lentulus* was previously described well by Hong *et al.* [14].

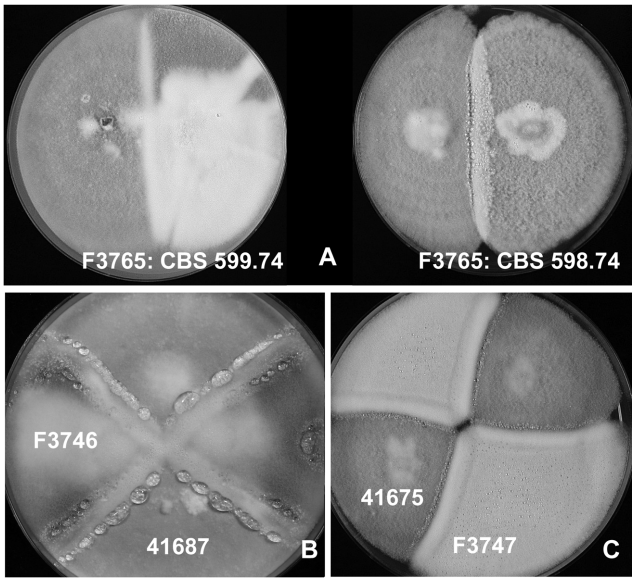

Fig. 2. Mating behavior of Korean strains of *Neosartorya fennelliae*. A, Mating of Korean Agricultural Culture Collection (KACC) F3765 with type strains of *N. fennelliae* CBS 598.74^T (MT A) and Centraalbureau voor Schimmelcultures (CBS) 599.74^T (MT a), KACC F3765 produced ascomatal with CBS 598.74 (MT A); B, Ascomata formation between KACC F3746 and 41687, C. ascomata formation between KACC 41675 and F3747.

Heterothallic species in AsF. Twenty one strains produced ascomata only when mated with compatible strains (Fig. 2). Seventeen strains from 11 soil samples were identified as *N. fennelliae* on the basis of their mating behavior and β-tubulin gene sequence, and their mating types were denoted in Table 2. *N. fennelliae* was characterized by its ascospores, which evidenced two equatorial crests and shallow rugose to reticulate convex surfaces (Fig. 1). Colonies of the species on CYA and MEA evidenced diverse patterns depending on the strains, usually with minimal to sparse conidiation, and the strains often evidenced a light yellowish color on the obverse and reverse of the CYA and MEA plates. Although 17 strains produced ascomata when mated with compatible strains in *N. fennelliae*, the intraspecific heterogeneity was high. KACC 41687, CBS 598.74^T, and CBS 599.74^T were separated from the other strains, evidencing a minimum of 97.8% similarity with the others (Fig. 3). However, the intraspecific group of the three strains was not supported by calmodulin gene phylogeny (data not shown).

Four strains from Korean soil generated ascomata when mated with *N. udagawae* CBS 114218^T (= KACC 41156), thereby suggesting an identification of *N. udagawae*. They were also clustered into the *N. udagawae* group in the β-tubulin tree (Fig. 3). The species evidenced lenticular ascospores with two equatorial or several irregular crests and tuberculate convex surfaces (Fig. 1). The species were found in only two soil samples and the strains of this study were composed of only one mating type.

Homothallic species in AsF. *N. quadricincta* was well separated from the other homothallic species in AsF, as it evidenced ascospores with four distinct equatorial crests. Nine strains from four soil samples were identified as *N. quadricincta* on the basis of ascospore morphology (Fig. 1) and β-tubulin gene sequence (Fig. 3). The species was characterized by comparatively slower growth on CZA (< 30 mm 7 days at 25°C) than on MEA, and ascospores with 4 prominent equatorial crests and convex surfaces harboring raised flaps of tissue or long ridgelines.

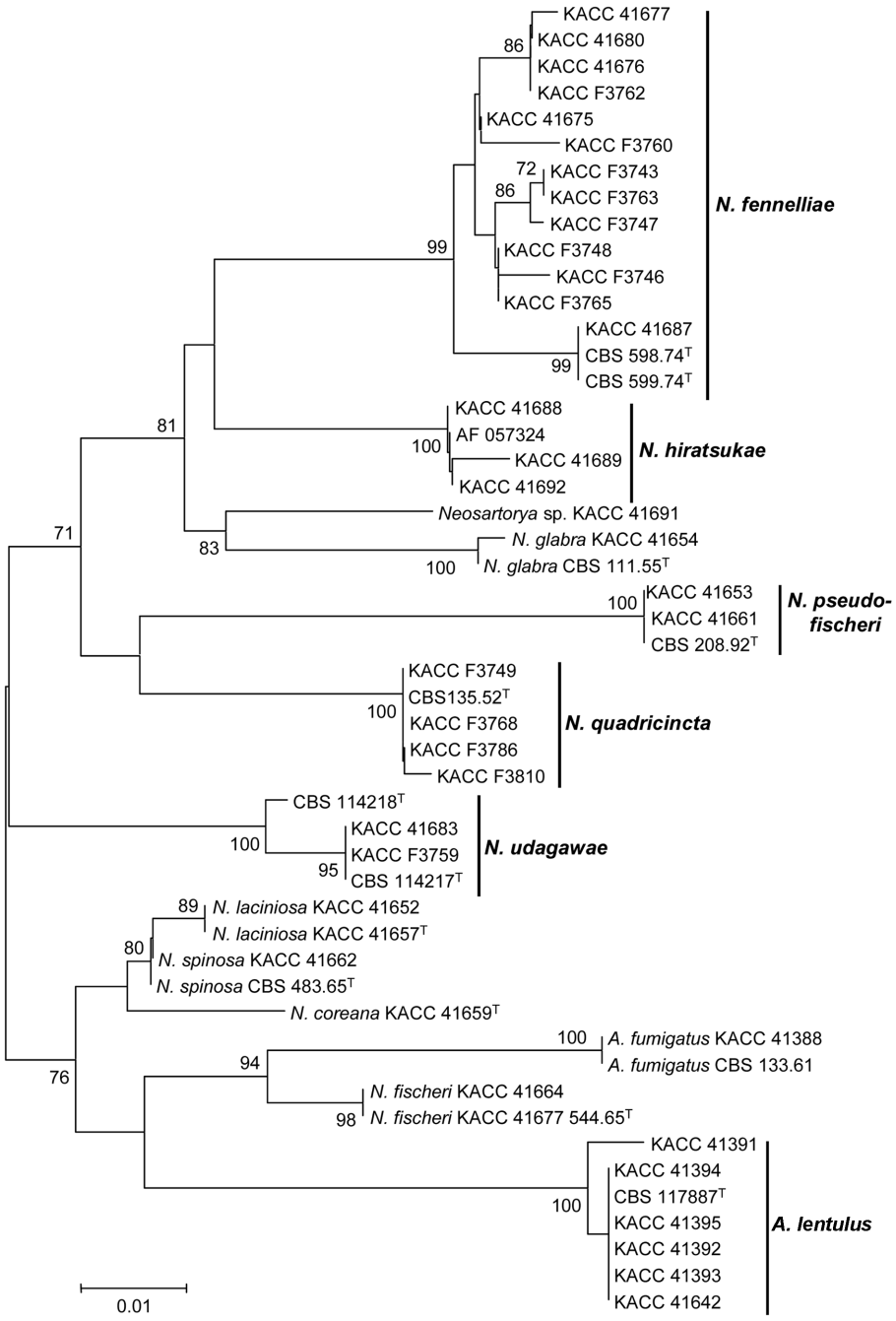

Fig. 3. Taxonomic position of Korean strains of *Aspergillus* section *Fumigati* based on partial β-tubulin phylogeny. Partial β-tubulin gene (primers bt2a and bt2b) sequences were first analyzed using the Tamura-Nei parameter distance calculation model with gamma-distributed substitution rates, which was then used to construct the Neighbor-Joining tree with MEGA version 3.1. The numbers above or below the nodes represent bootstrap values of > 70% (out of 1,000 bootstrap replications). KACC strains were isolated from arable soil in Korea.

Neosartorya fischeri and *N. hiratsukae* in homothallic AsF species in Korea had ascospores with two closely appressed crests and reticulate convex surfaces. Two strains were identified as *N. fischeri* based on their ascospore morphology (Fig. 1) and β-tubulin gene sequence (Fig. 3). The species was characterized by ascospores with convex surfaces bearing coarse and irregular height networks. Five strains from three soil samples were identified as *N. hiratsukae* based on their ascospore morphology (Fig. 1) and β-tubulin gene sequences (Fig. 3). The species was characterized by very restricted growth on CZA (< 12 mm) and ascospores with convex surfaces bearing fine and irregular height networks.

Species evidencing ascospores with two distinctly sepa-

rated equatorial crests were *N. glabra*, *N. pseudofischeri*, *N. spinosa*, *N. laciniosa*, and *N. coreana* in AsF in Korea. Their species differentiation was described by Hong *et al.* [13]. Four strains were identified as *N. glabra*, because they had ascospores with smooth convex surfaces and with relatively rigid equatorial crests (Fig. 1). β-Tubulin phylogeny supported this identification (Fig. 3). The five strains from five soil samples were identified as *N. pseudofischeri*, because they evidenced ascospores ornamented with raised flaps of tissue, in the form of triangular projections or long ridgelines (Fig. 1). This convex surface ornamentation was similar to that of *N. quadricincta*, and both shapes were somewhat roseate. However, *N. pseudofischeri* had two equatorial crests, whereas *N. quadricincta* had four crests. The identification of *N. pseudofischeri* was confirmed by the β-tubulin gene sequence tree (Fig. 3). *N. spinosa*, *N. laciniosa*, and *N. coreana* were isolated from four, two, and one soil samples, respectively, and the morphological and molecular characteristics of the Korean strains were previously described in detail by Hong *et al.* [13]. KACC 41691 was similar to *N. pseudofischeri* in terms of its ascospore morphology, but its phylogenetic position differed from that of *N. pseudofischeri* (Fig. 3). This may possibly represent a new species in AsF, but further examination will be required in order to clarify the taxonomic position of the strain.

AsF flora in arable soil. AsF species usually arrest attention in the fields of medical and food mycology. Only two species in AsF--*A. fumigatus* and *N. fischeri*--were described in the Compendium of Soil Fungi [16]. In this study, it is interesting to note that 13 extraordinary AsF species were isolated with high frequency from 17 arable soil samples. *A. fumigatus*, *A. lentulus*, *N. fennelliae*, and *N. quadricincta* were isolated with particularly high frequency. This may suggest that AsF species are widely distributed in arable soils in Korea and might perform important roles in crop growth.

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